HOW DOES APOPTOSIS OF OOCYTES AND GRANULOSA CELLS DUE TO CIGARETTE SMOKE EXPOSURE TO MICE BALB/C ? : EXPRESSION SMAD3, GDF9, APOPTOSIS

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ABSTRACT
Cigarette smoke contains harmful toxins for body, including nicotine, tar, and carbon dioxide gas which are carcinogens. This content is harmful for health of active and passive smokers, including disturbances in the reproductive system, hormone secretion disorders, and impotence. The nicotine contained in cigarette smoke is one of the ingredients that increase free radical levels in the body. Free radicals cause an increase in ROS compounds in the body2,3,4. Smoking affects various metabolic and biological processes in the body including hormone secretion, this is due to the increased effect of oxidative stress due to the increase of free radicals in the body caused by cigarette smoke and blood serum. It will also affect the production of FSH and LH by the pituitary in the brain5. Cigarettes are a process of tobacco using additional ingredients or not. Cigarettes with added ingredients are called kretek cigarettes while cigarettes without any addition are called white cigarettes. In smoking process, there are two reactions, namely the combustion reaction and the pyrolysis reaction. The combustion reaction with oxygen will form CO2, H2O2, NO, SO, and CO compounds5. Pyrolysis reactions are reactions that break down chemical structures into many chemical compounds with complex structures6,7. Cigarette smoke enters the respiratory tract. NOX is an enzyme in all cell membranes. Hot steam contained in cigarette smoke that enters the air then will react with NOX become NADPH oxidase and O2 (superoxide). By SOD, the superoxide radical (O2-) is catalyzed into hydrogen peroxide (H2O2) and oxygen (O2). H2O2 is systemic oxidant that can affect all cells in the body4, H2O2 which is increased due to cigarette smoke will also penetrate the ovaries and affect granulosa cells. In granulosa cells, estrogen is produced. Estrogen in granulosa cells will affect

INTRODUCTION
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the growth and development of oocytes through transcription activity by SMAD3. After activation of estrogen receptors7,8,10 H2O2 in cells can damage DNA, fat, and protein. Smad 3 is protein that mediates the transcriptional activity of estradiol in granulosa cells that will affect the development of oocytes after activation of estrogen receptors10. If Smad 3 protein synthesis is inhibited, it cannot activate estrogen so that it cannot affect the growth and development of granulosa cells, even though granulosa cells are one of the cells that play a role in folliculogenes. If granulosa cells do not develop, then folliculogenes will also be disrupted11. H2O2 will also affect the proteins found in oocyte cells. In oocytes, there is GDF-9 which is glycoprotein secreted by oocytes, and an important factor for oocyte growth. Besides that GDF9 can stimulate granulosa cell proliferation. The deficiency of GDF-9 will cause the development of follicles stopped, there is no cells around the follicle, and ability of the oocyte to divide miosis will be reduced13,14. Smoking can also cause oxidation of glutathione (GSH, an antioxidant that protects DNA from damage caused by ROS), lowers blood levels of antioxidants, and increases the release of superoxide radical15. If the low GSH and GSSG increased, the oxidant H2O2 increased, then it can damage the cells that will undergo apoptosis protein16.GSH functions as anti-apoptosis, if GSH is low, then BAX will increase, Bcl2 decreases, Cyt c is oxidized and released, releasing caspase 9 and caspase 3 there will be apoptosis. But if the GSH is high, then the BCL2 increases, the cyt c will not be oxidized and released so that there is no apoptosis.17
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MATERIALS AND METHODS

The type of research was analytically using True Experimental design with post-test control group design. Using sample of 20 female Balb/c mice (Mus Musculus) Balb/c aged around 8-10 weeks with bodyweight of 25-30 grams. The independent variable was exposure to cigarette smoke, while the dependent variable was Mad 3, GDF9, granulosa cell apoptosis, and oocyte apoptosis. This study was divided into 2 groups: Group 1: control group without exposure to cigarette smoke, Group 2: treatment group with exposure to cigarette smoke a dose of 1 cigarette per day for 20 days using a smoking pump. This research was conducted in Embryology Department, Faculty of Veterinary Medicine, Airlangga University. Data analysis used independent t-test or Mannwithney.

RESULTS AND DISCUSSION

Examination of Smad 3 expression in granulosa cells, GDF 9 in oocytes, using the IHC method. Examination of granular cell apoptosis and oocyte apoptosis using the TUNEK Essay method. The results showed that exposure to cigarette smoke can decrease Smad 3 expression in granulosa cells, decreased GDF9 expression in oocytes, increased granulosa cell apoptosis, but did not affect oocyte apoptosis. The results of the examination are listed in the table 1.

<table>
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<th>Variables</th>
<th>Group</th>
<th>Mean</th>
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Comparison of the mean results of Smad 3 and GDF9 expression between the control and treatment groups. Table 1 showed the mean Smad 3 and GDF9 expressions for each group. The lowest mean of Smad 3 and GDF9 expressions were found in the treatment group, while the highest mean was found in the control group. In the data normality test using Shapiro Wilk, it was found in the control group that data was normally distributed (p = 0.110) and treatment group data was normally distributed (p = 0.321). Then proceed with homogeneity test of the data using Levene test, and data was homogeneous (p = 0.06). The results of statistical tests using independent t-test obtained p = 0.000 <0.05, so it can be concluded that there is an effect of cigarette smoke exposure on the expression of Smad 3 and GDF9 in oocyte cells of Balb/c mice.

The lowest mean of apoptotic index expression in granulosa cells was found in the control group, while the highest mean was found in the treatment group. In the data normality test using Shapiro Wilk, it was found in the treatment group that data was normally distributed (p = 0.783). The results of statistical tests using Mann Whitney obtained p = 0.000 <0.05, so it can be concluded that there is an effect of cigarette smoke exposure on the apoptosis index in granulosa cells of Balb/c mice.

Examination of oocyte apoptosis in the control group and the treatment group, there was no apoptosis in the oocyte. The results of statistical tests using the Mann Whitney test obtained p = 1 > 0.05, so it can be concluded that there is no effect of cigarette smoke exposure on the apoptosis index in oocytes of Balb/c mice.
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Figure 1 showed that in control group Smad 3 expression in granulosa cells was positive, it was not seen in the cigarette smoke exposure treatment group that Smad 3 expression in granulosa cells is negative. Deficiency of Smad3 in mice will decrease fertilization because Smad3 plays role in follicle growth, aggression, and differentiation. Administration of FSH to mice will express normal FSHR. This indicates that the interaction between Smad3 and FSH signals is involved in downstream FSHR in mouse ovaries. The Smad signal pathway is critical for the transmission of the TGF-β signal family from cell surface to the nucleus. In the nucleus, Smad functions as a co-modulator of the transcription process for the regulation of TGF-β-dependent gene expression. Activin and inhibin are members of the TGF-β family. The difference between SMAD2 and SMAD3 proteins lies in the N-terminal region of MAD homology 1 (MH1), while SMAD2 contains 30 amino acids that are not in Smad3. The Smad2δexon3 protein occurs naturally due to the loss of this amino acid. Excessive expression of Smad2δexon3 will stimulate FSHβ mRNA in the same amount as stimulated by Smad3. Taken together, transcription activation of FSHβ will be stimulated by Smad3 and Smad2 in mouse gonadotroph cells. Other evidence of Smad's involvement in FSH regulation can be seen from its role in the folliculogenesis process. The process of folliculogenesis itself is primarily responsible of FSH hormone so that the involvement of Smad in FSH regulation will indirectly affect the folliculogenesis.

Figure 1. Smad3 expression, GDF9 expression, granulosa cell apoptosis, and oocyte apoptosis
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CONCLUSION
It can be concluded that cigarette smoke also decreased Smad3 expression in granulosa cells, SMAD 3 is a protein that mediates the transcription activity of estradiol in granulosa cells which influences oocyte development after estrogen receptor activation. Besides, exposure to cigarette smoke also increased apoptosis in granulosa cells. The presence of apoptosis in granulosa cells can cause folliculogenesis disorders in the ovaries. Cigarette smoke also decreased the expression of GDF 9 in oocytes, but there was no difference in the apoptosis in oocytes. It was possible that through necrosis, vacuolar was seen in oocytes.

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CONFLICT OF INTEREST
The authors state that there are no conflicts of interest regarding the publication of this article.

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This study was approved by the institutional review board of Ethical Approval (2/KE/037/02/2019). The research received a certificate from the Airlangga University.

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